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Current Opinion in Plant Biology

Control of cell proliferation by microRNAs in plants

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Plants have the ability to generate different and new organs throughout their life cycle. Organ growth is mostly determined by the combinatory effects of cell proliferation and cell expansion. Still, organ size and shape are adjusted constantly by environmental conditions and developmental timing. The plasticity of plant development is further illustrated by the diverse organ forms found in nature.MicroRNAs (miRNAs) are known to control key biological processes in plants. In this review, we will discuss recent findings showing the participation of miRNA networks in the regulation of cell proliferation and organ growth. It has become clear that miRNA networks play both integrative and specific roles in the control of organ development in *Arabidopsis thaliana*. Furthermore, recent work in different species demonstrated a broad role for miR396 in the control of organ size, and that specific tuning of the miR396 network can improve crop yield.

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Current Opinion in Plant Biology 2016, **34**:68–76

This review comes from a themed issue on **Cell Biology**

Edited by **Keiko Sugimoto** and **Arp Schnittger**

For a complete overview see the Issue and the Editorial

Available online 26th October 2016

http://dx.doi.org/10.1016/j.pbi.2016.10.003

Introduction: versatile regulation of plant development by miRNAs

Small RNAs are key regulators of gene expression in animals and plants [reviewed in [1,2]]. Depending on their biogenesis pathways, they are usually classified in different groups such as small interfering RNAs (siRNAs), natural antisense transcript derived siRNAs (nat-siRNAs), transacting-siRNAs (ta-siRNAs) and miRNAs [1,2]. Plant development notoriously relies on several evolutionary conserved miRNAs networks, as well as on the ta-siRNA pathway. MiRNA biogenesis requires the cleavage of a non-coding RNA harboring a fold-back precursor by a ribonuclease type III called DICER-LIKE1 (DCL1) [2,3,4]. The released miRNAs have around 21 nucleotides and function in the context of a complex containing an ARGONAUTE (AGO) protein, generally AGO1 [5]. The miRNA guides the complex to target RNAs that have base complementarity to the miRNA. Target mRNAs can be translationally inhibited or cleaved [1,6]. The biogenesis of ta-siRNAs depends on the cleavage of a *TAS* RNA by an AGO complex guided by a miRNA, which is then turned into double stranded RNA by RNA-dependent RNA polymerase 6 (RDR6) that is finally cleaved by DCL4 to generate the small RNAs that will also become incorporated into AGO complexes [1,2].

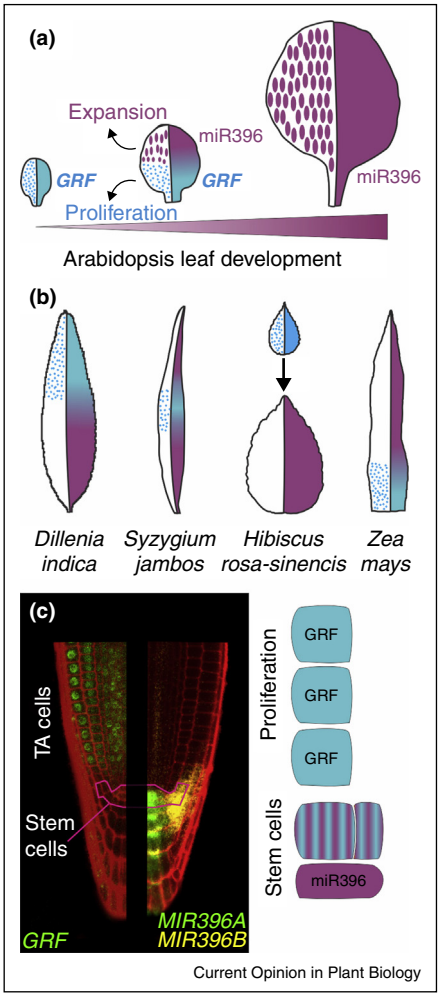
Plant miRNAs are generally encoded by their own transcriptional units and, like regular protein-coding genes, have promoters recognized by RNA polymerase II [1]. In addition, miRNAs are usually encoded by small gene families whose members might generate identical or nearly identical small RNAs [7], but can be differentially expressed. Therefore, miRNAs can be spatio-temporally regulated to be expressed in the right cells and at the right time. In this way, miRNAs can either fine-tune target gene expression or erase completely the targeted transcripts. Here, we provide an update on the role of microRNAs in the regulation of organ growth, focusing on the control of cell proliferation in tissues actively engaged in cell division such as young leaves and root meristems. Although most work has originally been carried out in the model plant *Arabidopsis thaliana*, recent studies extend and validate the biological roles of miRNA networks to other species including crops of agronomical importance.

Control of organ growth by miRNAs

Many of the evolutionarily conserved miRNAs regulate transcription factors involved in plant growth and development. The above-ground parts of plants derive from the activity of stem cells located in the shoot apical meristem (SAM), and the establishment and maintenance of the SAM requires the action of several miRNA networks such as miR394, miR171, and miR165/6 [8,9].

Leaves originate at the flanks of the shoot apical meristem. Early steps in the development of the leaf primordia involve the functional separation from the meristem as well the establishment of a dorso-ventral axis that leads to the establishment of the two sides of the leaf, the adaxial (upper side) and the abaxial (lower side) side, which will be specialized in light capture and gas exchange, respectively. Several small RNAs already participate in these early patterning events, including transacting small RNAs and miRNA networks, which have been reviewed recently [10,11].

Figure 1



Definition of cell proliferation domains by the miR396/GRF regulatory network. **(a)** Leaf growth in Arabidopsis. Distribution of cell proliferation and expansion indicated by silhouettes of leaves at different developmental stages. Light blue dots indicate mitotic cells and magenta ellipses, expanding cells. MiR396 expression domain is indicated in magenta, while GRFs are expressed in light blue colored regions. Note that at intermediate stages of leaf development cell proliferation and *GRF* expression are restricted to the proximal part of the leaf, while cell expansion occurs in the distal part. **(b)** Distribution of cell proliferation, GRF (light blue) and miR396 (magenta) expression in different plant species with various leaf growth polarities. In *Zea mays*, cell proliferation is restricted to the base of the leaf, while in *Dillenia indica* cell proliferation is concentrated in the apical part. Besides, cell proliferation in the middle of the leaf, presumably supplying cells both to the apical and basal halves of the leaf occurs in *Syzygium jambos*. In *Hibiscus rosa-sinensis*, cell proliferation is evenly distributed, and stops at the same time in all regions when cells start to elongate. In all cases, expression and cytological studies suggest that miR396 is expressed in post-mitotic cells, restricting the *GRF* expression to the cell proliferation domain of the developing leaf. **(c)** The left panel shows the expression pattern of miR396 coding genes (*MIR396A* and *MIR396B*) and *GRF3* in Arabidopsis root tips. The images were obtained by Laser Confocal Microscopy of plants transformed with the corresponding reporters after cell walls were stained with propidium iodide to visualize the cellular organization of the root meristem. The image showing the expression pattern of the miR396 coding genes is an overlay of two confocal microscope images showing the expression of *MIR396A* (colored in green) and *MIR396B* (yellow). The right panel is a scheme that summarizes the expression patters of miR396 and *GRFs*, and the orientations of divisions in the region of the root stem cells (magenta) and transit-amplifying cells (TA cells, light blue). MiR396 is expressed at the highest levels in stem cells excluding GRF from this region. In turn, GRF expression in the TA cells represses the expression of stem cell genes and stem cell-like behavior of TA cells.

Cells located in the leaf primordia will engage in active cell proliferation and the leaf will finally develop into a relatively flat organ with a defined size and shape. During this process, miRNA networks control and coordinate cell proliferation affecting the overall organ or specific leaf domains, while integrating external and developmental signals.

Promotion of cell proliferation in leaf growth

Cell proliferation occurs first throughout the smallleaf primordium in Arabidopsis. However, once the organ begins to develop, most of the proliferative cells become restricted to a defined region at the base of the organ. At this stage, cells located at the distal part of the organ begin their expansion. Afterwards, the proliferative domain at the proximal region of the leaf disappears rather abruptly and, cells in this region also begin to expand (Figure 1a) [12,13].

The *GROWTH-REGULATING FACTORs* (*GRFs*) are plant specific transcription factors defined by the presence of conserved WRC (Zinc Finger) and QLQ protein domains, which promote leaf growth [14,15]. Seven out of the nine Arabidopsis *GRFs* have a target site for miRNA miR396 [16]. This miR396-GRF network is conserved at least in angiosperms and gymnosperms [17].

Ectopic expression of miR396 in Arabidopsis causes a significant reduction of *GRF* expression and results in smaller leaves with reduced cell number [18,19,20]. In contrast, plants harboring *GRF* genes with mutations in the miR396-binding site that render the target resistant to the microRNA activity (*rGRFs*) have a higher level of the corresponding *GRF* transcript, an increased number of leaf cells and ultimately bigger leaves [19,21].

MiRNA miR396 begins to accumulate in the zone harboring expanding cells at the distal part of the developing leaf repressing the expression of the GRFs outside the area of proliferative cells (Figure 1a). A miR396 gradient along the longitudinal axis of the organ, with higher expression at the distal part, generates an opposing gradient of the GRFs [17,19,22•]. At later leaf developmental stages and coincidental with the cessation of cell proliferation, miR396 is expressed throughout the organ while the *GRFs* are turned off [17,19,22•].

Still, ectopic expression of the GRFs causes a delay in leaf senescence which can be uncoupled from the control of cell proliferation indicating that the GRFs also perform functions beyond organ growth [21]. Furthermore, studies on GRF7 have shown that this specific miR396-regulated *GRF* is involved in the abscisic acid and osmotic stress responses [23].

In addition to their post-transcriptional control by miR396, the GRFs form a protein complex with GRF-INTERACTING FACTORs (GIFs), such as ANGUSTIFOLIA3 (AN3)/GIF1, which are considered to be transcriptional co-activators [15,24]. Interestingly, AN3 also interacts with components of chromatin remodeling complexes such as the SWI/SNF ATPase BRAHMA [21,25•,26•], and it has been suggested that AN3 can link the chromatin remodeling machinery to the GRFs [25•].

A survey of leaf growth in 75 eudicot species identified species with different growth polarities [27•]. While in many cases growth was located at the base of the developing leaves like in *Arabidopsis thaliana*, other patterns were also identified, including stronger growth at the center or distal part of the organ, or even uniform growth throughout the organ [27•]. In these divergent patterns of organ growth, there was a correlation with the pattern of miR396/GRF expression: while the GRFs were located in the region of the leaf actively engaged in cell proliferation, the miRNA miR396 accumulated in the other parts of the organ (Figure 1b) [27•]. It is however not known whether a change in the positional expression of the miR396 and the *GRFs* is sufficient to specify the cell proliferation region or, alternatively, which are the upstream events leading to their different expression patterns in species with different growth polarities.

A similar correlation pattern can be even found in monocotyledonous leaves, such as maize and rice, with the GRFs being preferentially expressed in the division zone at the base of the leaf (Figure 1b) [28,29]. Furthermore, the overexpression of a miR396-resistant *GRF1* in maize also resulted in longer leaves due to an increase in the size of the basal division zone [26•].

Repression of cell proliferation in leaf growth

MiR319 is another evolutionarily conserved miRNA that regulates a group of *TCP* (*TEOSINTE BRANCHED1*, *CYCLOIDEA*, *PCF1* and *2*) transcription factors. In contrast to the *GRFs*, the miR319-regulated *TCPs* repress cell proliferation [22•] and trigger cell differentiation [30,31,32••]. In turn, controlled expression of the miR319-TCP module can generate different leaf sizes and shapes [30].

TCP4 binds directly to the promoter of *MIR396B*, one of the two Arabidopsis genes encoding miR396, and mutations in the TCP4-binding box of the *MIR396B* promoter significantly decrease its expression in leaves (Figure 2) [22•]. Part but not all the activity of TCP4 as a repressor of cell proliferation can be explained by the induction of miR396. In principle, TCP4 can activate several different pathways that inhibit cell proliferation. On the one hand, it has been shown to up-regulate genes involved in the biosynthesis of jasmonic acid, a hormone known to inhibit cell proliferation [33]. On the other hand, TCP4 can directly regulate ICK1 [22•], an inhibitor of cyclin-dependent kinases and core component of the cell cycle machinery that arrests cell proliferation [34], and modulates auxin [35] and cytokinin pathways [32••].

MiR159 is similar in sequence to miR319, however it regulates GAMYB transcription factors [36]. Loss of function of *mir159a* and *mir159b* causes the ectopic expression of *MYB33* and *MYB65*, which in turn affects plant development and reduces cell proliferation in leaves [37,38]. However, *MYB33* and *MYB65* are not implicated in normal leaf development and miR159 functions to reduce the GAMYB transcripts to inconsequential levels in leaves [39]. This contrasts with miR319, which quantitatively modulates *TCP* expression [30,33,35].

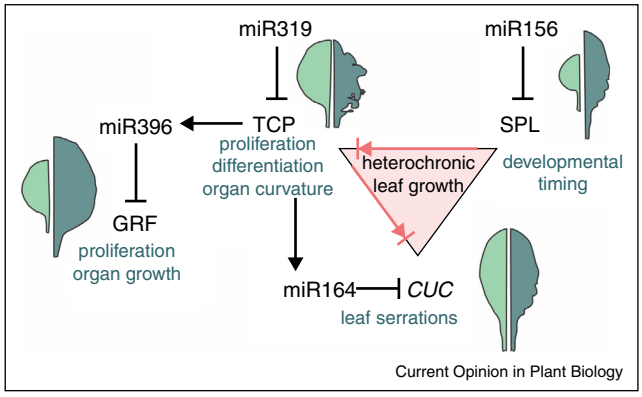
Elegant studies have shown that the balance of the miR319/TCP network also controls floral organ growth in Arabidopsis [40], and a loss-of-function mutant of *MIR319a* has stunted sepals and stamens and narrow petals [40]. In inflorescences, where both TCPs and MYBs are expressed, they have been shown to form a protein complex and act in flower maturation [41]. In turn, TCP4 binds directly to the promoter of *MIR167A*, a miRNA that represses *ARF6/8* [41].

Three *ARF* genes, *ARF2*, *ARF3* and *ARF4*, are post-transcriptionally regulated by ta-siRNAs, and the regulation of *ARF3* and *ARF4* has been linked to the establishment of leaf polarity [reviewed in [10,42]]. The biogenesis of the ta-siRNAs regulating *ARFs* requires the specific activity of the evolutionarily conserved miR390, which is associated to AGO7 before the action of RDR6 and DCL4 [43]. Analysis of *ARF2* has shown that it is a repressor of cell proliferation and loss-of-function mutations in the gene result in significantly larger leaves and seeds [44,45].

Sculpting leaf margins by miRNAs

The strong overexpression of miR319, as seen in the *jaw-D* mutant, or *TCP* down-regulation generates changes in leaf curvature and the formation of crinkles due to an excess of cell proliferation in the leaf periphery (Figure 2). Studies in a snapdragon mutant in the *CINCINNATA* gene, which is a *TCP4* homolog with a miR319-binding site, have shown a delay in the repression of cell proliferation, especially at the leaf margins [46]. In *Arabidopsis thaliana*, crinkles are seen after the simultaneous down-regulation of several redundant TCPs [30,33,47].

Figure 2



Interplay of evolutionary conserved microRNA-transcription factor networks during leaf development.
The functions associated to each miRNA network are indicated as well as silhouettes of leaves representing the phenotypes observed in Arabidopsis after modifying each network. The pink triangle refers to the protein-protein interactions between the miRNA targets of the different networks.
MiR396 targets *GRF* transcription factors, which promote cell proliferation and organ growth. An increase of GRF activity results in larger leaves. MiR319 inhibits class II TCP transcription factors that inhibit cell proliferation and promote differentiation. Significant changes in TCP levels affect organ curvature. TCP4 directly activates the promoters of miR396 and miR164. MiR164 targets *CUC* transcription factors whose activities contribute positively to the generation of leaf serrations. MiR156 targets SPL transcription factors which promote the phase change from the juvenile to the adult phases of vegetative development and also to reproductive development; but they also influence leaf growth itself.
The miR156, miR319 and miR164 networks are further interconnected by the protein-protein interactions of their targets (indicated by pink triangle). During the development of the younger leaves miR156 levels are high and therefore SPL levels are low, and TCP proteins form dimers with CUC proteins (indicated by a red arrow) which in turn leads to smooth margins. Later on in development, when miR156 levels go down, SPL levels increase and the TCP-CUC dimers are replaced by TCP-SPL dimers (indicated by red arrow). The released CUC proteins dimerize and leaf serrations are formed.

The TCPs regulate the expression of *ARR16* and cytokinin responses, which have been implicated in the development of crinkles [32••]. The emerging picture links the miR319-regulated transcription factors with several hormone pathways indicating their participation in many biological process. Furthermore, miR319-regulated TCP4 interacts with the SWI/SNF ATPase BRAHMA to regulate target genes linking the miR319 network with the chromatin remodeling pathway [32••].

Arabidopsis leaves have serrations at the margins. The balance between miR164 and its targets, the transcription factors of the NAC class (NAM, ATAF1/2, CUC2) significantly affects the serrations of Arabidopsis leaves, especially through the relationship between *CUC2* and *MIR164A* (Figure 2) [48,49]. The leaf serrations develop through the local control of cell proliferation regulated by the miR164-*CUC* network [50]. In turn, miR319-regulated *TCP* transcription factors have been shown to regulate the expression of *MIR164* promoters, therefore linking the two miRNA networks (Figure 2) [35]. Leaf serrations are further modified by the miR160 regulatory networks that control ARF transcription factors [51].

Interestingly, the miR319 and miR164 regulatory networks that in *Arabidopsis thaliana* control the leaf margins and the periphery of the organ, are also involved in the development of compound leaves by other species such as tomato [52,53,54]. Actually, these networks can quantitatively affect leaf complexity by determining the morphogenetic window during which leaflets can be formed [52,53,54].

Integration of developmental and environmental signals by miRNA-regulated transcription factors

Leaf size and shape are affected by the developmental timing of the plant. A key developmental decision is the transition from the juvenile to adult phase of the plant, a process that is regulated by miR156, which controls *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) transcription factors [55,56]. In *Arabidopsis thaliana* this vegetative phase change is associated with differences in organ development including the generation of larger leaves with more cells, an increase in leaf serrations, and changes in the length/width ratio of the blade (Figure 2) [55,56,57,58].

Recent work indicates that protein-protein interactions of transcription factors regulated by miRNAs are at least partially responsible for these changes. In Arabidopsis, juvenile leaf margins are smooth. Direct interaction between CUC2 as well as CUC3 and TCP4 has been shown in yeast two hybrid and bimolecular luminescence complementation assays, and it has been suggested that these dimers prevent the formation of the active CUC2-CUC3 dimers that lead to serrations (Figure 2) [59••]. TCP4 also forms heterodimers with SPL9, a target of miR156 which is expressed preferentially in early stages of plant development. Once miR156 levels decrease and SPL9 levels increase it is possible that the formation of TCP4-SPL9 dimers allows the formation of active CUC2-CUC3 complexes leading to serrated leaves (Figure 2). This idea is strengthened by the observation that SPL9 negatively influences the formation of CUC-TCP4 dimers in yeast three hybrid assays [59••]. Future work will need to confirm the presence of the different heterodimers *in vivo* and how their balances shift and influence leaf growth and shape.

Leaf growth is also influenced by environmental conditions such as light quality, abiotic stress and nutrient availability. Under unfavorable conditions there is a

reduction in cell number and leaf size [60,61]. Experiments with UV-B irradiation have shown that a reduction of cell numbers in Arabidopsis leaves is caused by the induction of miR396 and the repression of the GRFs [62]. Plants expressing *GRF* transcription factors insensitive to miR396 grow larger leaves despite UV-B irradiation [62]. The induction of miR396 by UV requires the activity of MPK3, however the mechanisms underlying the activation of miR396 remain unknown.

SAP11 is a secreted protein effector from phytoplasma, which interacts and destabilizes miR319-regulated TCPs [63]. Overexpression of SAP11 causes a similar phenotype to the overexpression of miR319 [63]. This inactivation of TCP transcription factors by SAP11, and the further modification of the TCP controlled pathways has been linked to the success of the pathogen [63]. It would be interesting to know whether the evolutionary conserved miRNA networks are also conserved targets for modification during abiotic and biotic stress in different species.

Definition of cell proliferation domains by the miR396-GRF regulatory network in roots and leaves

Overexpression of miR396 has been shown to affect many organs in Arabidopsis. Flowers from Arabidopsis plants overexpressing miR396 display reduced numbers and size of all organs, unfused or missing carpels, short ovule integuments, defective gametogenesis and even organs with mosaic identity [64,65], while leaves can also have polarity defects [20,66]. Although high levels of miR396 are associated with repression of organ growth, it is unclear whether the different effects observed by miR396 overexpression are caused by the same mechanism.

In contrast to the defined leaf growth, the Arabidopsis root grows in an indeterminate fashion with a root meristem constantly supplying new cells for organ growth [67]. The size of the root meristem is regulated by the interplay between auxin and cytokinin [68]. Interestingly, the latter pathway recruits the miR165/166 regulatory network which regulates HD-ZIP class III transcription factors [69]. This network is well known for its key roles in other biological processes including in root patterning [70], the formation of the shoot apical meristem and the establishment of leaf polarity [71].

Expression studies in Arabidopsis roots have shown that miR396 genes are expressed in the stem cell niche (Figure 1c) [72••]. PLETHORA (PLT) transcription factors, which are master regulators of root development, have a peak of expression that establishes the root stem cells [73,74], where they also control miR396 levels, especially *MIR396A* [67,72••].

In turn, the *GRFs* are expressed in transit amplifying cells of the root meristem [72••], which is a group of actively proliferating cells derived from the stem cells that are already committed to differentiate. As the stem cells divide slowly, transit amplifying cells sustain organ growth [75]. Therefore, in both, roots and leaves, *GRF* expression co-localizes with the region that contains cells actively proliferating [14,15,19,27•,72••].

Overexpression of miR396 represses root growth in Arabidopsis, although the cellular cause for this repression is different than in leaves. The concomitant depletion of GRFs causes the transit amplifying cells to acquire stem cell properties, activating the expression of genes usually detected in root stem cells, including *PLT* transcription factors *per se* (Figure 1c) [72••]. Furthermore, transit amplifying cells in the root proliferate through anticlinal amplificatory divisions, while miR396 overexpressors divide slower, and frequent periclinal cell divisions are observed, which are typical for stem cells. As a consequence, miR396 overexpression increases the size of the root meristem [72••], which contrasts to the reduction in the size of the shoot apical meristem in the same plants [19]. Therefore, while in a developing leaf the miR396-GRF domains stablish the boundaries between proliferation and differentiation, in roots the miR396-GRF network contributes to the stem cell- cell proliferation boundary.

Improving crop yield through modifications in miRNA networks

Several microRNAs have been shown to be expressed during embryo and seed development, and repression of targets by miRNAs is essential to avoid the precocious activation of differentiation pathways and enable proper embryo patterning [76]. Seeds are the ultimate product of important crops, and both grain size and number are major components that determine yield. Three independent studies in rice recently identified a natural occurring allele of *OsGRF4*, a rice GRF regulated by miR396, that increases the size of grains, that are formed by larger embryos, endosperm and husks [77•,78•,79••]. These differences are in part due to an increase in cell proliferation as the grain husks contain a larger number of cells, but are also due to an increase in cell size. Plants with larger grain size have two mutations in the miRNA-binding site of *OsGRF4* that impair the repression by miR396 [77•,78•,79••]. Therefore, a natural *GRF* gene resistant to miR396 activity, similar to those artificially designed in Arabidopsis [19,21], enhances grain size in rice.

In addition, blocking miR396 activity using target mimicry results in an increase in the number of rice spikelets [80••], further suggesting this miRNA network might be exploited for crop improvement. The mechanisms underlying the increase of grain size by GRFs in rice are currently unknown, although it has been suggested to be

linked to exacerbated responses to brassinosteroids [79••] and changes in auxin metabolism and signaling [80••].

In addition to the increase of seed size in rice, blocking miR396 increases fruit size in tomato [81], suggesting a general role of the miR396 regulatory network in controlling organ size. Furthermore, modification of the miR396 network has been associated with resistance to cyst nematode infection in Arabidopsis roots [82] and the efficiency of mycorrhizal associations [83].

Yet, not every modification resulting in higher levels of GRF transcription factors is beneficial for plant yield. Overexpression of ZmGRF10 [84] inhibits organ development, while miR396-resistant *GRF1* [26•] in maize affected female fertility and reduced crop yield. Precise expression patterns of the GRFs as well as their protein sequence seem to play a role in the final outcome of their activity. In Arabidopsis, microRNA resistant versions of the *GRF* transcription factors, like *rGRF3*, result in a larger increase of leaf size [21] compared to *rGRF2* [19], *rGRF7* [23] or *rGRF9* [20]. Furthermore, *rGRF3* expressed from its own promoter sequences results not only in an increase of leaf size, but also in a delay in senescence. These effects can be uncoupled by controlling the timing of *rGRF3* expression using promoters that are active in specific developmental stages [21].

Conclusion

Yield depends on organ size and plant architecture. Understanding the mechanisms that regulate cell proliferation and organ growth might then provide efficient means to improve plant productivity. The evolutionary conserved miRNA networks described here provide a framework for rational design of better harvests with larger seeds for crop production or bigger leaves for improved light capture.

Plant development in general and organ growth in particular are broadly controlled by miRNA networks. In turn, these networks seem to be interconnected through cascades of transcription factors controlling miRNAs which in turn control other transcription factors. Furthermore, unrelated transcription factors regulated by different miRNAs can also interact at the protein level. Still, the molecular link between these miRNA transcription networks and core components controlling the cell cycle are currently unknown in most cases. Understanding the regulatory logic of these networks and the molecular mechanisms by which they control organ growth will be a challenge for the near future.

Most interesting, results obtained in the model plant *Arabidopsis thaliana* describing the biological functions and mechanisms of miRNA networks have proven to be useful to predict and understand their roles in other plant species, even in those with direct agronomical importance.

Acknowledgements

We thank members of the Palatnik lab for discussions. The work in small RNAs in the lab is supported by grants from the Argentine Ministry of Science, ANPCyT (PICTs) to RER, CS and JP.

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- • of special interest
- •• of outstanding interest

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